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SV2C IS A SYNAPTIC VESICLE PROTEIN WITH AN UNUSUALLY RESTRICTED LOCALIZATION: ANATOMY OF A SYNAPTIC VESICLE PROTEIN FAMILY

R. JANZ* and T. C. SÜDHOF†

Center for Basic Neuroscience, Department of Molecular Genetics, and Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX 75235, U.S.A.

Abstract—We describe here the identification and molecular characterization of a new brain protein that we named SV2C because it is homologous to the synaptic vesicle proteins SV2A and SV2B, and because it is also recognized by the monoclonal SV2 antibody that led to the initial discovery of SV2A and SV2B. SV2C is more closely related to SV2A (62% identity) than to SV2B (57% identity), and contains 12 transmembrane regions similar to these proteins. To characterize SV2C and compare its properties and localization with those of SV2A and SV2B, we raised an SV2C-specific antibody. Using this antibody, we show that SV2C is an N-glycosylated protein that is concentrated on small synaptic vesicles; in addition, it is found on microvesicles in adrenal chromaffin cells. We evaluated the relative localization of the three SV2 isoforms by staining rat brain sections with antibodies specific for SV2A, SV2B and SV2C. Analysis of the resulting staining patterns confirmed previous conclusions that SV2A is ubiquitously expressed in virtually all synapses. SV2B, although more restricted in distribution, was also found in a wide variety of synapses throughout the brain. In striking contrast to this general localization and to similarly wide distributions of other synaptic vesicle proteins, SV2C was observed only in few brain areas. High levels of SV2C were found primarily in phylogenetically old brain regions such as the pallidum, the substantia nigra, the midbrain, the brainstem and the olfactory bulb. SV2C was undetectable in the cerebral cortex and the hippocampus, and found at low levels in the cerebellar cortex.

Our data suggest that closely related members of a synaptic vesicle protein family can either have very general (SV2A) or restricted distributions (SV2C), possibly in order to allow specialization in the regulation of the expression or of the function of these abundant synaptic vesicle proteins. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: synaptic vesicle, glycoprotein, transporter, SV2, SVOP.

SV2 is a glycoprotein that was initially identified by a monoclonal antibody raised against synaptic vesicles, and was shown to be present on synaptic vesicles and on endocrine secretory vesicles in all vertebrates studied.[7] Molecular cloning elucidated the structures of two SV2 isoforms, SV2A and SV2B. SV2A and SV2B both react with the monoclonal SV2 antibody, are highly homologous to each other and are both present on synaptic vesicles. Both isoforms contain 12 potential transmembrane spanning domains and a large intravesicular loop that includes three N-glycosylation sites.[3,4,8] SV2 proteins are among the most abundant and conserved components of synaptic vesicles in vertebrates, but no SV2 ortholog could be identified in invertebrates. Even the nearly completed *C. elegans* sequence does not include an SV2 protein, although it does encode a more distantly related protein, SVOP.[16] SVOP is a synaptic vesicle protein with 12 transmembrane regions similar to SV2's. In contrast to SV2, however, SVOP is conserved in all multicellular organisms studied and is less homologous to SV2A and SV2B than they are to each other.[16] SV2A, SV2B and SVOP exhibit significant homology to a large family of proteins that are transporter molecules for a variety of substrates. This led to the hypothesis that SV2 could be a vesicular transport protein for an unknown substrate. However, no transport activity has so far be demonstrated biochemically. Furthermore, the vesicular neurotransmitter transporters that have been characterized so far show no significant homology to SV2.[18,20,27] Therefore, although it is possible that SV2 proteins are transporters, their functions are unclear.

The distributions of SV2 proteins in brain and endocrine tissues were studied using immunocytochemistry and *in situ* hybridizations.[2,7,13,28,29] These experiments revealed that SV2 proteins are present on all small synaptic vesicles independent of transmitter type. SV2A is expressed ubiquitously throughout the brain, while SV2B is present in a more restricted pattern in a subset of synapses. In neuroendocrine cell lines like PC12 cells, only SV2A but not SV2B was detected. Interestingly, only SV2B was observed in pinealocytes, where it was found on small microvesicles. In the retina, SV2B is concentrated in ribbon synapses of the outer plexiform layer, whereas SV2A is found mainly on convential synapses in the inner plexiform layer. The presence of SV2A and SV2B in synaptic vesicles with different transmitters makes it unlikely that they serve as vesicular neurotransmitter transporters. If SV2 proteins are in fact transporters, it is more likely that they transport something, which is important for synaptic vesicles and large dense-core granules, independent of transmitter type. An alternative hypothesis is that SV2 proteins are not transporters but have evolved from transporters to perform another function in synaptic vesicles.

In the present study, we describe the cloning and characterization of a novel, third isoform of SV2, which we named SV2C. Interestingly, SV2C is also present on synaptic vesicles like SV2A and SV2B, but exhibits a very restricted distribution. It is only present on a small subset of synapses in

*Present address: Department of Neurobiology and Anatomy, UT Houston Medical School, Houston, TX 77030, U.S.A.

†To whom correspondence should be addressed. Tel.: + 1-214-648 1876; fax: + 1-214-648 1879.

E-mail address: TSudh@mednet.swmed.edu (T. Südhof)

Abbreviations: EST, expressed sequence tag; NMDA, *N*-methyl-D-aspartate; PAGE, polyacrylamide gel electrophoresis; PNGaseF, peptide-*N*-gycosidase F; SDS, sodium dodecyl sulfate; SV, synaptic vesicle protein; TMR, transmembrane region.

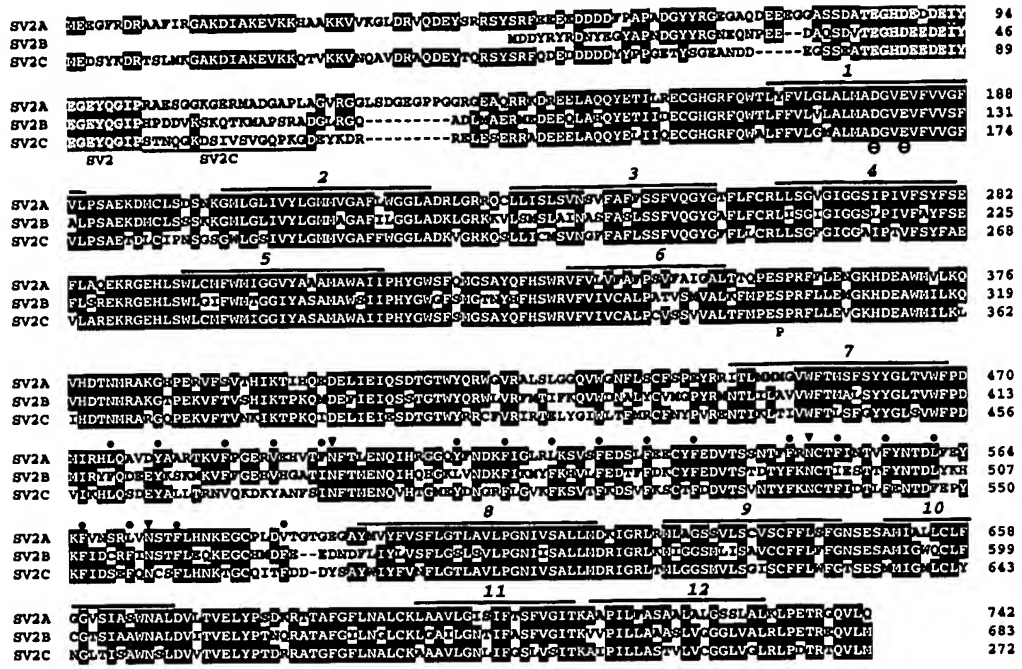

Fig. 1. Structure of SV2 proteins. The sequences of rat SV2A, SV2B and SV2C are aligned for optimal homology. Sequences are identified on the left and numbered on the right. Identical residues are shown in white on a black background. At the N-terminus, the likely epitope sequence for the SV2 monoclonal antibody is highlighted with a grey background, and the adjacent non-conserved sequence used to generate an SV2C-specific peptide antibody is underlined and labeled SV2C. The 12 putative TMRs are numbered and are identified by lines above the sequences. The conserved negatively charged residues in TMR 1 are shown on a grey background and labeled with a − symbol below the sequence. The potential protein kinase C phosphorylation site between TMRs 6 and 7 is marked with a P. In the loop between TMRs 7 and 8, conserved phenylalanine residues are marked by bullets (·) and N-glycosylation consensus sequences by triangles.

phylogenetically old brain areas. Our data show that the SV2 family is more complex than anticipated previously and includes proteins that are either widely expressed in all synapses or restricted to a small subset of synapses.

EXPERIMENTAL PROCEDURES

Cloning of the complementary DNA of SV2C and construction of expression constructs

Databank searches identified a potentially novel SV2-related sequence that was also found in an expressed sequence tag (EST) clone (IMAGE *n* 656245; see Results). A ~300-bp fragment from this EST clone was used as a probe to isolate three distinct SV2C clones from a rat brain λZAP cDNA library. cDNA clones were sequenced directly and by subcloning of fragments into M13 vectors using an automated sequencer from ABI with the dye terminator technique. One cDNA clone contained the full-length coding region of SV2C and was used to generate a mammalian expression vector (pCMV-SV2C) by subcloning a 2.7-kb BssHII/SalI fragment from that clone into the MluI/SalI sites of pCMV5.[1]

Antibodies

A peptide corresponding to residues 187–210 of SV2C (Fig. 1) with an additional N-terminal cysteine (CSTNQGKDSTVSVGQPKGDEYKD) was coupled to keyhole limpet hemocyanin as described[17] and used for generation of rabbit antisera. Sera were affinity purified using peptide immobilized on agarose with the SulfoLink® kit (Pierce). The purified antibody was used at a dilution of 1:2000 for blots and for immunohistochemistry. The monoclonal antibodies against SV2,[7] synaptotagmin I (Cl41.1) and *N*-methyl-D-aspartate (NMDA) receptor 1 (Cl40.1), were a kind gift of Dr R. Jahn (Göttingen) and were used at a dilution of 1:2000. Monospecific polyclonal rabbit antibodies against SV2A and SV2B were a kind gift of Dr E. Link (Göttingen) and were used at a dilution of 1:1000. Secondary peroxidase-coupled goat anti-rabbit or rabbit anti-mouse antibodies were from Cappel and were used at a dilution of 1:10,000 for the enhanced chemiluminescense (ECL™) reaction. Immunohistochemistry of adult rat brain was performed as described in Rosahl *et al.*[22] using adult Sprague–Dawley rats (Harlan). Peptide blocks were performed by adding 10 mg/l peptide to the incubation reaction with the specific antibody.

Protein analysis

Subcellular fractions of rat brain proteins and bovine adrenals were prepared as described in Janz *et al.*[16] The protein concentrations of all fractions were determined using the Commassie Protein assay from Biorad and the concentrations were adjusted by dilution. Synaptic vesicles purified by controlled pore glass chromatography[15] were a kind gift of Dr R. Jahn (Göttingen). Deglycosylation with peptide-*N*-gycosidase F (PNGaseF) was done as described.[16]

Cell culture

COS cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Transfections were done using DEAE–dextran with chloroquin and a 2-min glycerol shock, as described by Gorman,[12] with 6.6 μg of DNA for 900,000 cells in a 10-cm dish. The cells were incubated for 72 h after transfection. For protein analysis, cells were washed once with phosphate-buffered saline, harvested in sodium dodecyl sulfate (SDS) sample buffer with a rubber policeman, and lysed by shearing 10 times using a syringe with a 25-gauge needle.

Northern blots

A 675-bp EcoRI/BamHI fragment of SV2C cDNA covering amino acids 187–410 of the coding region was labeled with α[^{32}P]dCTP and used as probe on a rat multi-tissue northern blot (Clontech) according to the manufacturer's protocol. The blots were washed for 10 min with 2×standard saline citrate at room temperature and then for 2×30 min at 55°C in 0.1×standard saline citrate/0.1% SDS and exposed for autoradiography at −70°C with an enhancement screen for various time periods.

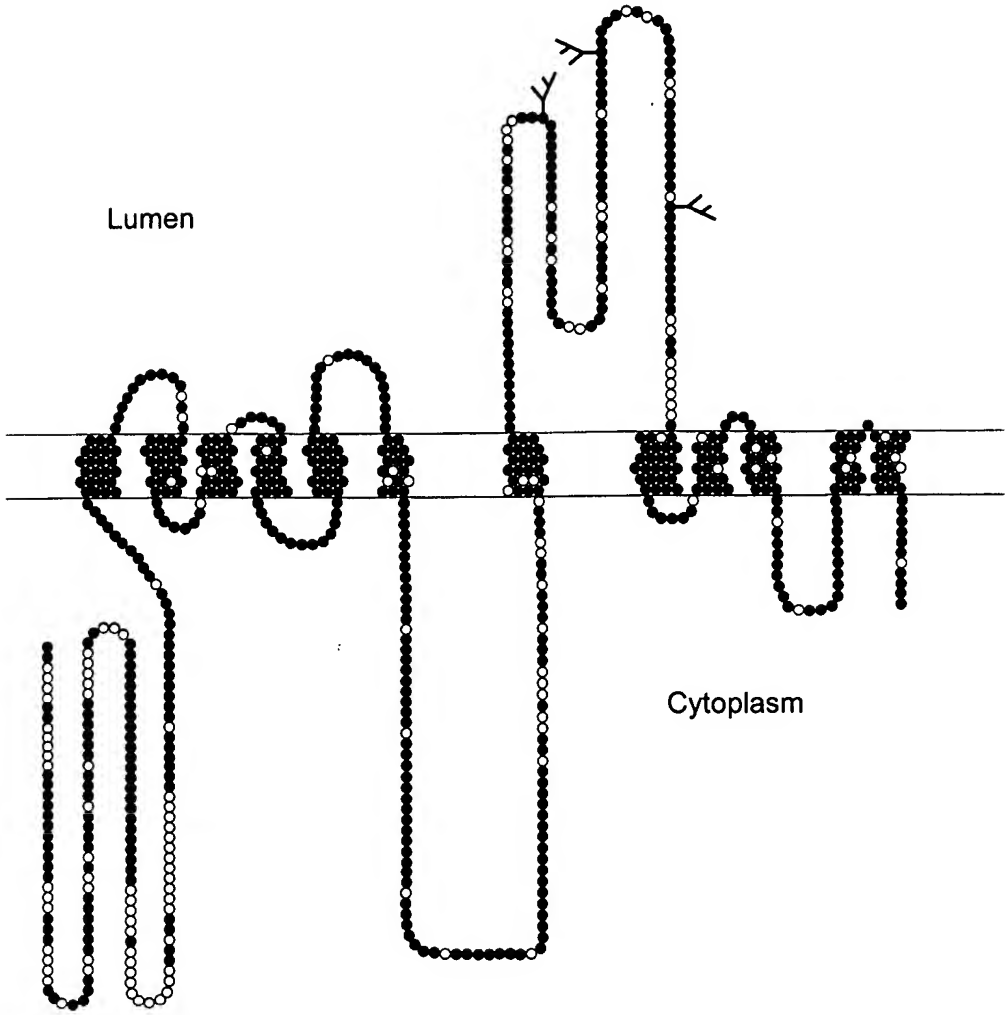

Fig. 2. Domain model of SV2 proteins: localization of conserved residues. Each residue in SV2 proteins is represented by a circle; filled circles represent conserved residues in all SV2 isoforms. Grey filled circles are residues conserved in two of the SV2 isoforms and open circles are non-conserved residues. The topology shown is deduced from sequence analyses which predict 12 TMRs with N- and C-terminal cytoplasmic regions. Note the relatively large conserved cytoplasmic loop between TMRs 6 and 7, and the relatively non-conserved large intravesicular loop between TMRs 7 and 8 that is probably N-glycosylated at the positions marked.

RESULTS

Cloning of SV2C

In order to search for new members of the SV2 gene family, we searched GenBank for homologous genes. We found a highly homologous sequence which was part of a cDNA sequence deposited as a mouse tyrosine phosphatase (accession *n* U24700). However, in the tyrosine phosphatase sequence, the homologous sequence was in antisense orientation. To clarify this paradox, we used the SV2 homologous part of the mouse sequence to search for matching EST clones in the database. We found an orthologous EST clone (IMAGE clone *n* 656245) which was derived from a mouse kidney library. Using the EST clone, we isolated three overlapping clones from a rat brain cDNA library. One of the clones contained an open reading frame coding for a protein of 727 amino acids which exhibited 62% sequence identity with SV2A and 57% identity with SV2B (Fig. 1). Because of this high degree of homology, we named the new protein SV2C. The SV2C sequence shown here is probably full length, because at the N-terminus it is highly homologous to SV2A (Fig. 1). This conclusion is supported by the fact that the transfected protein has the same size as endogenous SV2C (see below). None of the clones contained tyrosine phosphatase sequences, suggesting that the SV2-like sequence in the mouse tyrosine phosphatase cDNA clone represents an unphysiological fusion.

Sequence analysis of SV2C

We aligned the sequences of the three SV2 proteins which each other to analyse the structure of SV2C in comparison with SV2A and SV2B. Analyses of the SV2A and SV2B sequences suggested that they contain 12 transmembrane regions (TMRs) with cytoplasmic N- and C-termini.[3] The homology between SV2C and the other SV2 proteins extends over the entire proteins, suggesting that SV2C also contains 12 TMRs and cytoplasmic N- and C-termini. This leads to the domain model shown in Fig. 2; the model also indicates the degree of conservation of various parts of the SV2 proteins. The domain model reveals that SV2 proteins are largely composed of TMRs with relatively short free N- and

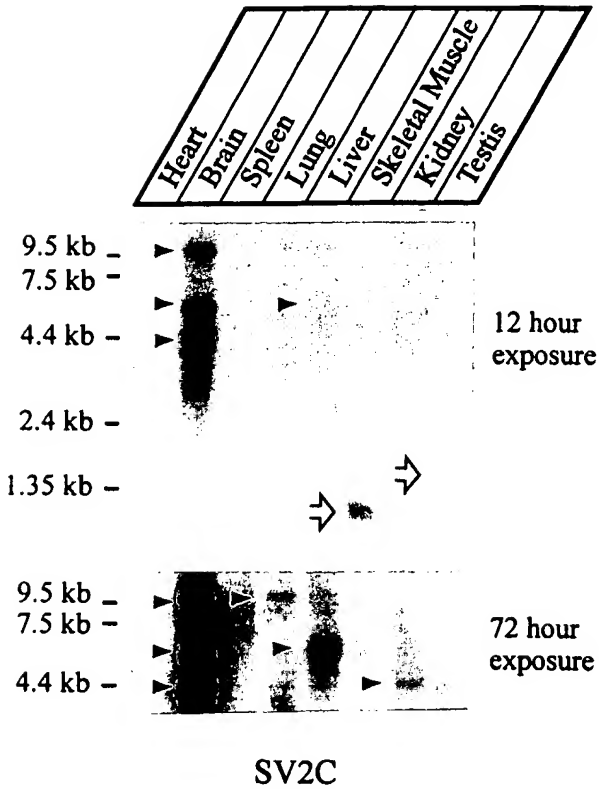

Fig. 3. RNA blotting analysis of SV2C expression. Blots are two different exposures of a rat multi-tissue RNA blot hybridized with an SV2C probe. Note that three brain-enriched mRNAs of approximately 4.5, 6 and 9.5 kB (arrowheads on left) are also expressed at low levels in other tissues. Open arrows in the top panel point to very small mRNAs that probably do not encode true SV2-related sequences. Numbers on the left indicate positions of molecular weight markers.

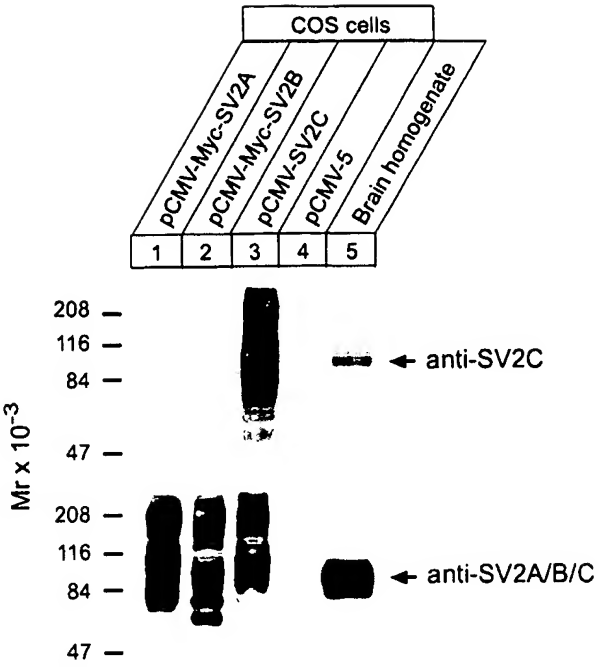

Fig. 4. Analysis of SV2C-specific antibodies. Protein from COS cells transfected with expression constructs for SV2A, SV2B and SV2C (lanes 1–3) or with a control vector (lane 4) were analysed together with rat brain homogenates (lane 5) by SDS–PAGE and immunoblotting. Blots were probed with an antibody raised to an SV2C-specific peptide (top panel) or the SV2 monoclonal antibody (bottom panel). Signals were visualized using ECL™.

C-termini. Most of the loops connecting TMRs are short except for the cytoplasmic loop between TMRs 6 and 7 and the intravesicular loop between TMRs 7 and 8 (Fig. 2).

The TMRs and the cytoplasmic loops connecting TMRs, including the large loop between TMRs 6 and 7, represent the most conserved SV2 sequences. The cytoplasmic N-terminal sequence and the large intravesicular loop constitute the least conserved sequences. The N-termini of SV2A and SV2C have very similar structures, whereas SV2B is shorter and quite different. Previous studies revealed that the SV2 monoclonal antibody recognizes an N-terminal epitope in SV2A and SV2B.[3,4] Since SV2C also reacts with the SV2 monoclonal antibody, it must also have this epitope. Comparison between the N-terminal sequences of the three SV2 proteins shows that there is only one highly conserved sequence, which is presumably the epitope (labeled SV2 in Fig. 1).

Although the intravesicular loop between TMRs 7 and 8 represents their least homologous sequence after the N-terminus, this loop shares several important characteristics in all SV2 proteins. There are three conserved N-glycosylation sites that are probably being used (arrowheads in Fig. 1; see below). An interesting feature of this loop is a repeat structure where every fifth residue has a strong preference for hydrophobic amino acids, especially phenylalanine (bullets in Fig. 1; starting with residue 475 in SV2A). This structure might represent a secondary structure that organizes the hydrophobic residues in a position where they interact with each other or other hydrophobic domains in the vesicular lumen. However, the five-residue periodicity is not consistent with a characterized secondary structure, making it difficult to predict the nature of the secondary structure assumed.

Analysis of the SV2 protein sequence for conserved potential phosphorylation sites revealed the presence of a protein kinase C consensus site (serine 357 in SV2A)[21] in the cytoplasmic domain between TMRs 6 and 7. It is possible that the activity of SV2 proteins is regulated by phosphorylation. Another conserved motif is the presence of an aspartate and a glutamate residue in the first TMR. The presence of these negatively charged residues is an indication that SV2 could transport a positively charged substrate.

Northern blot analysis of the expression of SV2C

To determine which tissues express SV2C, we hybridized a rat multi-tissue northern blot with an SV2C probe under high stringency conditions. Autoradiograms of such northerns with two different exposure times are shown in Fig. 3, demonstrating that SV2C is expressed in the brain by three major mRNAs of about 4.5, 6 and 9.5 kb (arrowheads). Although SV2C mRNAs are primarily synthesized in the brain, longer exposures of the blot revealed that they are also present in lung, liver and kidney (bottom panel in Fig. 3). Furthermore, smaller cross-hybridizing mRNAs of approximately 1–2 kb were observed in muscle and testis (open arrows). Most of the small mRNAs are too short to code for a full-length SV2 protein and probably represent artifacts. The expression of SV2C in non-neuronal tissues is consistent with the origin of the mouse SV2C EST clone from a kidney library.

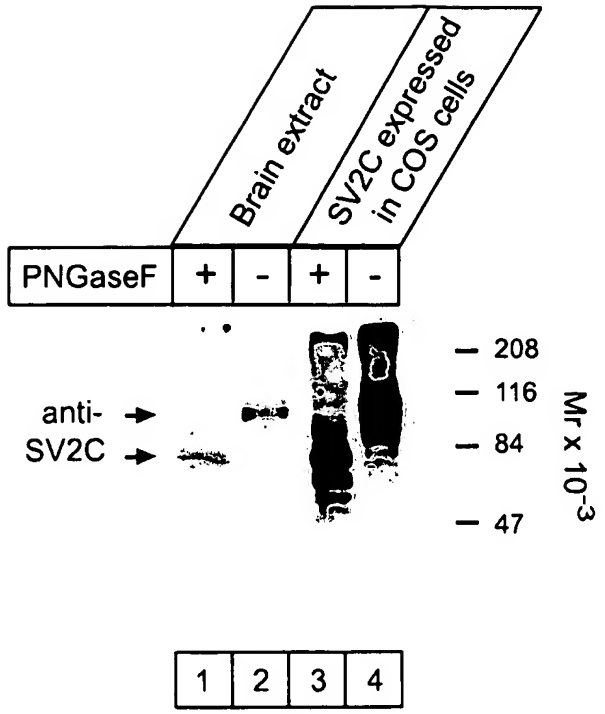

Fig. 5. SV2C is N-glycosylated. Homogenates of rat brain or COS cells protein transfected with SV2C were incubated with and without PNGase F, which cleaves N-linked sugars. Proteins were analysed by SDS–PAGE and immunoblotting with the SV2C-specific antibody.

Therefore, in contrast to SV2A and SV2B,[3] SV2C may also be expressed in non-neuronal tissues.

Generation of SV2C-specific antibodies

In order to study the protein that is encoded by the SV2C mRNA, we raised a polyclonal antibody against a synthetic peptide derived from residues 98–115 of SV2C. This sequence is not conserved in SV2A or SV2B (Fig. 1). The SV2C antibody was affinity purified, and its specificity was evaluated with transfected COS cells that synthesize SV2A, SV2B or SV2C (Fig. 4). When we analysed the transfected COS cells and rat brain proteins by immunoblotting with the SV2C antibodies, we detected a heterogeneous set of bands with mol. wts ranging from 9000 to 130,000 in COS cells transfected with SV2C, but not in COS cells transfected with SV2A or SV2B. Analysis of the same samples with the SV2 monoclonal antibody, however, revealed that all three types of transfections (SV2A, SV2B and SV2C) produced an immunoreactive band in COS cells, while control transfections did not (lower panel in Fig. 4). These data suggest that the polyclonal SV2C antibody is specific for SV2C, while the monoclonal antibody recognizes all three isoforms. After expression in COS cells, all SV2 isoforms migrated as a heterogeneous set of bands, possibly because of partial proteolysis, since COS cell extracts are very proteolytic, or because of incomplete glycosylation (see below).

In the brain, the SV2C-specific antibody detected a single band of approximately 95,000 mol. wt that is much less heterogeneous than the COS expressed bands but of approximately the same size (lane 5 in Fig. 4). The monoclonal antibody, in contrast, recognized several bands of approximately 90,000–100,000 which presumably represent mixtures of SV2A, SV2B and SV2C. A comparison of the signal intensity of the proteins in the brain extracts recognized by the SV2C-specific polyclonal and the general SV2 monoclonal antibody indicates that the amount of SV2C among the SV2 isoforms in the brain is relatively small compared to SV2A and SV2B.

SV2C is N-glycosylated

In order to analyse whether SV2C is a glycoprotein like SV2A and SV2B, we incubated homogenates of total brain and of COS cells expressing SV2C with and without PNGaseF, an enzyme that specifically cleaves N-linked sugars. Samples were then analysed by SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting with the SV2C antibody (Fig. 5). PNGaseF treatment resulted in a reduction of the apparent molecular weight of SV2C in

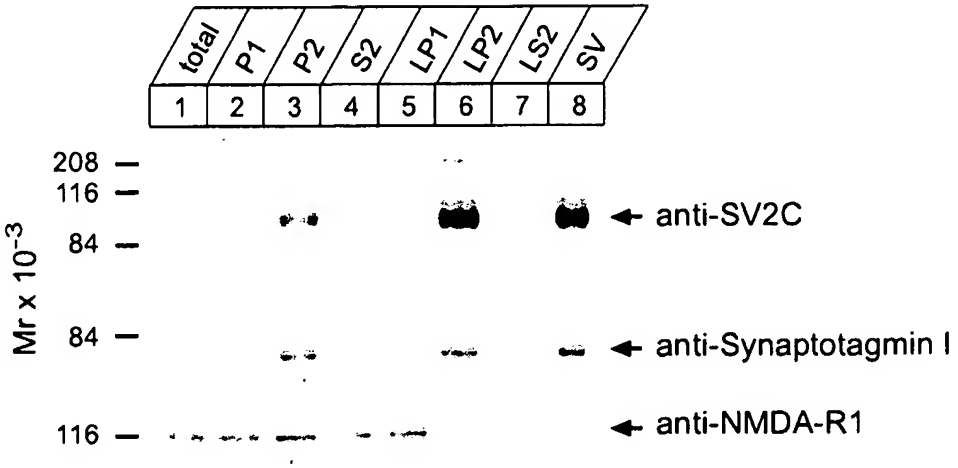

Fig. 6. SV2C co-purifies with synaptic vesicles. Rat brain homogenates (total; lane 1) were fractionated into a low-speed pellet P1 (lane 2), a synaptosomal pellet P2 (lane 3) and a supernatant of the synaptosomal fraction S2 (lane 4). Lysis of the synaptosomes yields a low-speed pellet LP1 (lane 5) which contains mainly synaptic plasma membranes, myelin and mitochondria, a high-speed pellet LP2 (lane 6) which is enriched in synaptic vesicles and the supernatant LS2 (lane 7) which contains soluble synaptosomal proteins. Synaptic vesicles were further purified by controlled pore glass chromatography (lane 8). Samples were analysed by immunoblotting with the SV2C-specific antibody (top panel), with antibodies to the synaptic vesicle protein synaptotagmin I (positive control, middle panel) and to the NMDA receptor I (negative control, bottom panel).

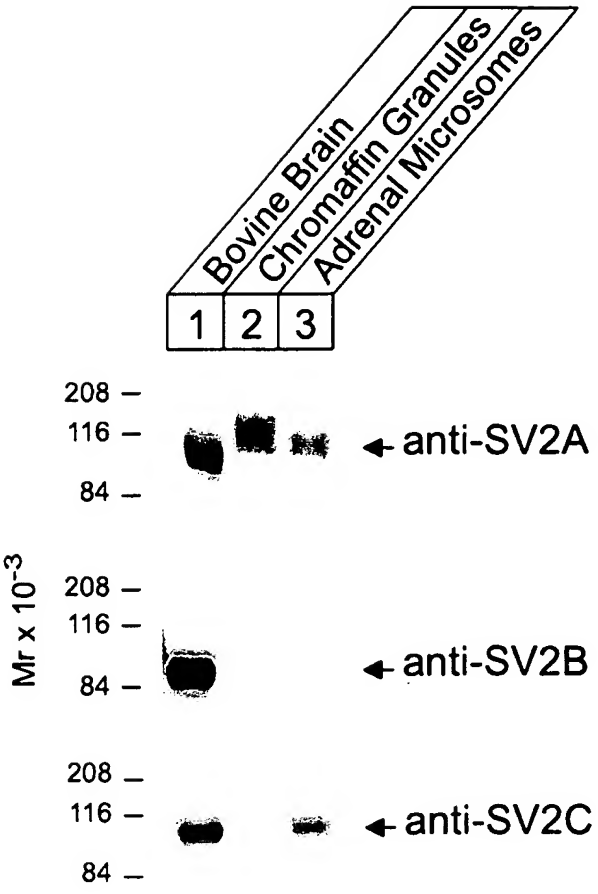

Fig. 7. SV2A, SV2B and SV2C in chromaffin granules and microsomes from the bovine adrenal medulla. Chromaffin granules and microsomes were purified from the bovine adrenal medulla and analysed by immunoblotting together with bovine brain homogenates. The samples were reacted with specific antibodies to all three SV2 proteins as indicated.

brain homogenates and in transfected COS cells from approximately 95,000 to 80,000. This result suggests that SV2C is N-glycosylated. After N-deglycosylation, SV2C still migrates as a heterogeneous set of bands in COS cells. This heterogeneity might be due to incomplete deglycosylation of the COS cell material, to other forms of processing in the COS cells like different forms of glycosylation that are not sensitive to cleavage by PNGaseF, or to partial proteolysis.

Subcellular localization of SV2C

Is SV2C also a synaptic vesicle protein like other SV2 proteins? To address this, we analysed subcellular fractions from rat brain by immunoblotting.[14,16] The highest levels of SV2C were found in LP2, the crude synaptic vesicle fraction obtained from hypotonically lysed synaptosomes, and in synaptic vesicles further purified by controlled pore glass chromatography (Fig. 6, lanes 6 and 8), suggesting that SV2C is a genuine synaptic vesicle protein. As a control for the purity of the fractions, they were analysed with antibodies to synaptotagmin I, a well-characterized synaptic vesicle protein which was used as a positive control. As expected and as observed for SV2C, synaptotagmin was also enriched in the synaptic vesicle fractions (Fig. 6). We then studied the distribution of the NMDA receptor, a postsynaptic protein that is not expected to be enriched in synaptic vesicles, as a negative control. The NMDA receptor is quantitatively excluded from the synaptic vesicle fractions, thereby confirming their purity (Fig. 6).

Localization of SV2 isoforms on adrenal chromaffin granules and microvesicles

SV2 was shown to be on adrenal chromaffin granules.[7] To explore if SV2B and SV2C are also present in the adrenal medulla and where they are localized, we used antibodies specific for SV2A, SV2B and SV2C. We then purified two fractions from the bovine adrenal medulla: chromaffin granules and microsomes containing synaptic-like microvesicles. These fractions were analysed together with bovine

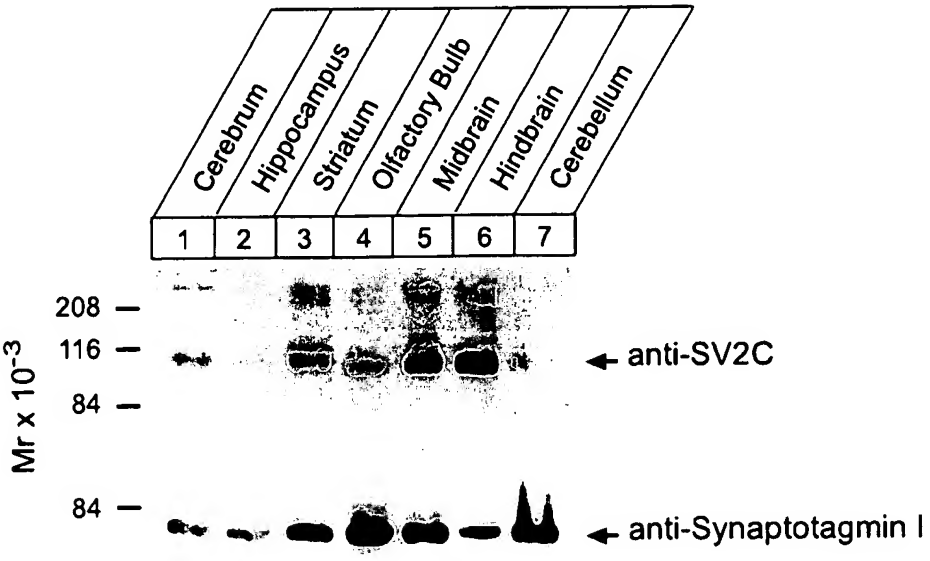

Fig. 8. Distribution of SV2C in rat brain regions. Panels show immunoblot analyses of proteins from the indicated areas dissected from rat brain. Blots were probed with antibodies specific for SV2C (top) and synaptotagmin I (bottom, positive control).

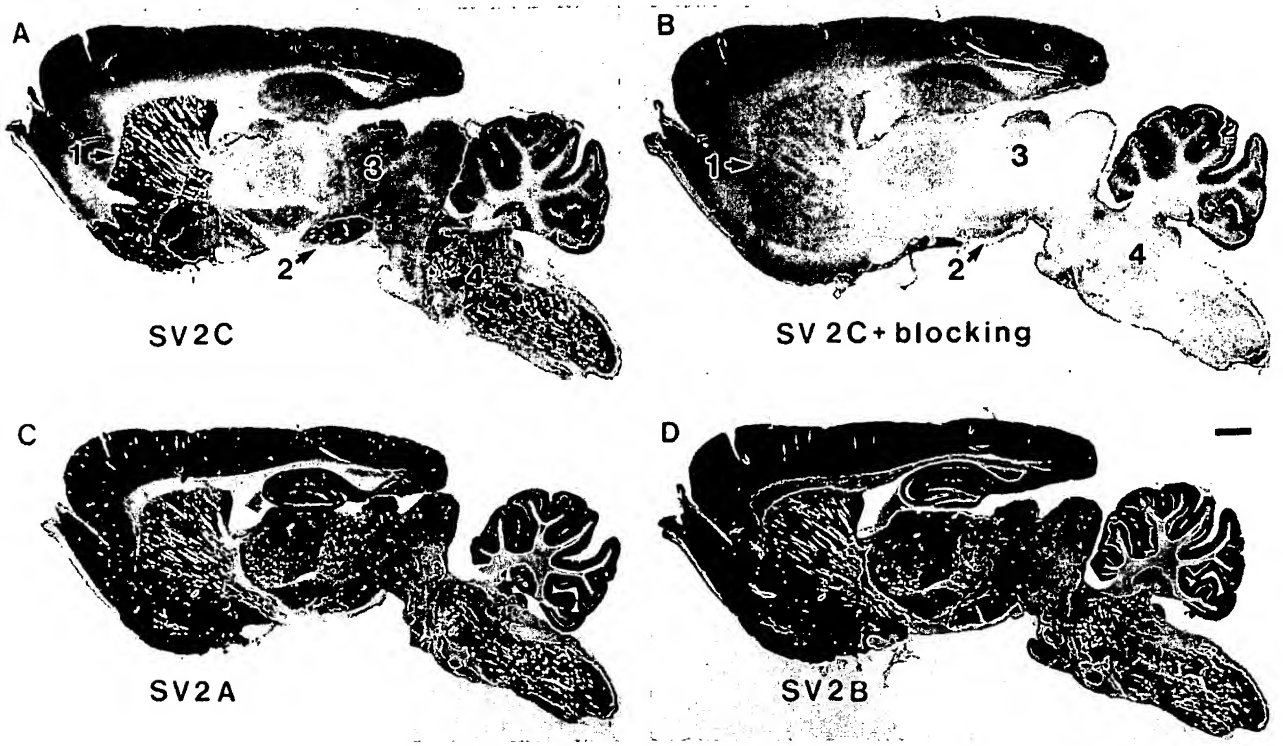

Fig. 9. Overall localization of SV2A, SV2B and SV2C in rat brain. Sagittal rat brain sections were probed with antibodies specific for SV2C (A), SV2A (C) and SV2B (D). Reactive areas were visualized by horseradish peroxidase staining followed by heavy metal enhancement. In B, the SV2C antibody was applied in the presence of the peptide used to raise the antibody as a specific blocking agent. Note the contrast between the broad distributions of SV2A and SV2B, and the restriction of strong specific staining for SV2C, which is only observed in the striatum (1 in A and B), substantia nigra (2), midbrain (3) and hindbrain (4). Scale bar = 1 mm.

brain homogenates (as a positive control) by immunoblotting (Fig. 7).

The blots show that SV2A is primarily present in chromaffin granules, with lower levels in microsomes. SV2B was not detected in the adrenal fractions. In contrast, SV2C was present at substantial levels in the microsomes but absent from chromaffin granules. These data suggest that, at least in the adrenal medulla, SV2A and SV2C are differentially localized. Many vesicle proteins are localized on adrenal synaptic-like microvesicles, some of which are enriched on the microvesicles while others are present only as trace components and reside primarily on large secretory vesicles.[5,9,16,19,25] The finding that only SV2A is found on large dense-core vesicles of chromaffin cells of the adrenal makes this organelle a potentially valuable system to study the function of this protein.

Localization of SV2C in various brain areas

We dissected different parts of the rat brain and analysed the amount of SV2C protein in the various fractions by SDS–PAGE and immunoblotting with the SV2C antibody (Fig. 8). As a control, the samples were also probed with the synaptotagmin I antibody. The following brain regions were dissected: cerebrum, striatum, hippocampus, midbrain, hindbrain (brainstem caudal of the midbrain, mainly pons and medulla oblongata) and the cerebellum. We found that SV2C is present at high levels in all areas of the central neuraxis, including the striatum, midbrain and hindbrain, and in the olfactory bulb. Only low levels of SV2C were detected in the cerebrum, the hippocampus and the cerebellum. In contrast to SV2C, synaptotagmin I was more evenly present in all of the brain areas analysed. These data suggest that SV2C is restricted to certain brain areas as opposed to the broad distributions of SV2A and SV2B.[2]

Immunohistological localization of SV2 proteins: overview

The biochemical data suggested that SV2C might have a quite different, more restricted distribution in brain than SV2A or SV2B. To test this, we performed immunocytochemistry. We applied the SV2C-specific antibody, as well as SV2A- and SV2B-specific antibodies, to sagittal sections of rat brain, followed by horseradish peroxidase-labeled secondary antibodies. Reactive areas were then visualized by horseradish peroxidase staining with heavy metal enhancement.[22]

The overall picture that emerges from these experiments is that SV2C is expressed at high levels in only very few brain areas (Fig. 9A), while SV2A and SV2B are widely present throughout the brain (Fig. 9C, D). SV2C is particularly highly expressed in the striatum, substantia nigra and pons/medulla oblongata (Fig. 9A). This staining pattern was specific, since it could be totally blocked by addition of the peptide used to raise the SV2C antibody (Fig. 9B). Most striking about the distribution of SV2C is that it appears to be almost completely absent from the neocortex, hippocampus and thalamus. Although some reactivity was observed in the neocortex, it was not diminished in the peptide block, suggesting that it represents background

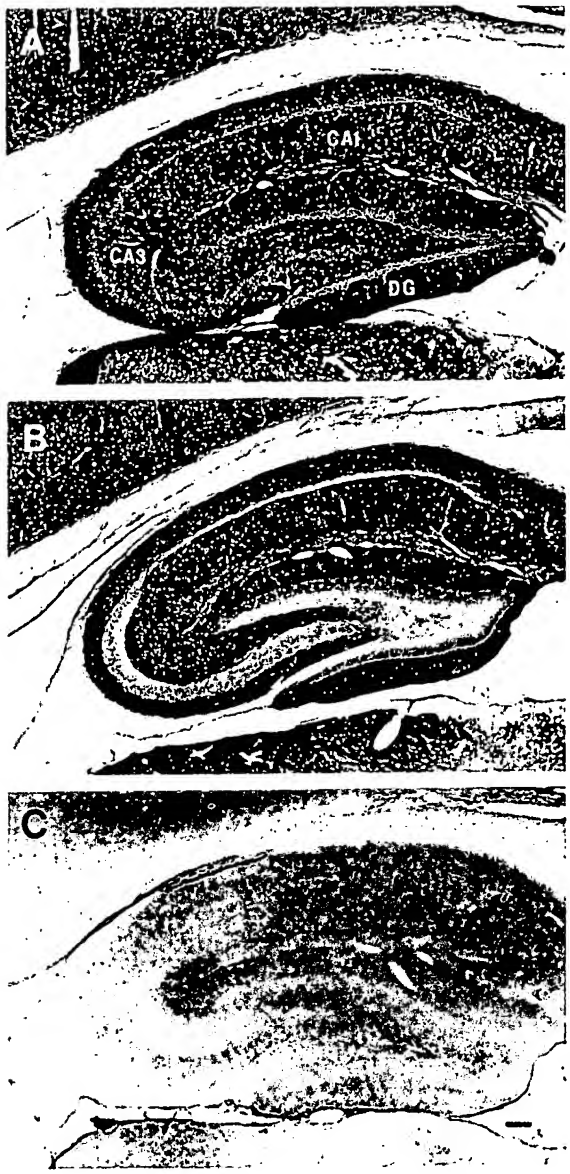

Fig. 10. Analysis of SV2A, SV2B and SV2C in rat hippocampus. Sections of rat hippocampus were stained with specific antibodies for SV2A (A), SV2B (B) and SV2C (C). In A, the CA1 and CA3 regions and the dentate gyrus (DG) are identified. Scale bar = 0.1 mm.

staining (compare Fig. 9A with Fig. 9B). In addition, there appears to be only very little staining of the cerebellar cortex. These results indicate that SV2C may be selectively excluded from cortical structures.

To confirm this striking result, we analysed the distribution of SV2A, SV2B and SV2C in the hippocampus in greater detail (Fig. 10). Immunoreactivity for SV2A was observed throughout the hippocampus. SV2B was present in most regions, except for those occupied by synapses that are formed by dentate gyrus granule cells, e.g., mossy fiber terminals in CA3. This analysis confirms previous results[2] that SV2A is ubiquitously present in all synapses, while SV2B is widely present in most, but not all, synapses. In contrast to these two widely distributed isoforms, however, we detected no staining for SV2C in any part of the hippocampus, even at higher magnification (Fig. 10C).

SV2 proteins in the cerebellum and underlying brainstem nuclei

We next studied the distribution of the SV2 forms in the cerebellum (Fig. 11). Again, SV2A and SV2B were found to be largely co-distributed in the molecular and granule cell layers of the cerebellar cortex. SV2C, by contrast, was detected only in discrete puncta in the granule cell layer; no specific staining of the molecular layer was observed (Fig. 11C). The SV2C-positive puncta in the granule cell layer probably correspond to individual synapses or clusters of synapses; their density is lower than the density of puncta stained with SV2A or SV2B antibodies, indicating that not all synapses contain SV2C. The absence of SV2C from the molecular layer agrees well with its absence from the neocortex.

In addition to the granule cell layer, SV2C was also observed in the deep cerebellar nuclei (Fig. 12D). These nuclei, as well as multiple brainstem nuclei, appear to contain relatively high levels of SV2C in addition to the other isoforms of SV2 (see area 4 in Fig. 9A). A high-magnification view of the brainstem nuclei reveals that most areas in the brainstem are positive for SV2C (Fig. 12E, F), in contrast to its very discrete presence in more rostral brain regions (Fig. 9A).

SV2C in the basal ganglia

The striatum and the substantia nigra probably represent the most intensely SV2C-labeled brain areas (Figs 9, 12). In the striatum, the highest levels of SV2C are found in the pallidum, with lower but still significant amounts in the putamen and the nucleus accumbens (Fig. 12). Reciprocal synaptic pathways connect the striatum and substantia nigra; the enrichment of SV2C in these areas indicates that SV2C could serve as a marker for these areas. Although it is not possible from the staining patterns to determine which type of synapses are stained with the SV2C antibody, consideration of all of the different staining patterns indicates that SV2C is not specific for a particular neurotransmitter system. For example, dopaminergic inputs are also received in the cortex,[11] which is negative for SV2C, suggesting that SV2C is not specific for dopaminergic neurons. Similarly, the staining pattern does not fit GABAergic terminals, since these are abundant in the thalamus and cortex. Thus, the distribution of SV2C is not coincident with a particular neurotransmitter system.

Localization of SV2 isoforms in the olfactory bulb

The analysis of the dissected brain parts for SV2C indicated a high level of SV2C in the olfactory bulb. This structure has two major synaptic layers, which are morphologically well defined. The olfactory nerve forms a synaptic connection with the mitral cell dendrites in the glomeruli. The mitral cells then form a specialized form of reciprocal denrodendritic synapse with the granular cells in the external plexiform layer.

We examined the distribution of SV2 proteins in the olfactory bulb in sagittal sections with SV2A-, SV2B-, and SV2C-specific antibodies (Fig. 13A, B, E). In addition, we stained adjacent sections for synapsin as a general synaptic marker (Fig. 13D). All three SV2 isoforms were found in both synaptic structures. However, compared to SV2A and SV2B and synapsins, SV2C was highly concentrated in the external plexiform layer, indicating that SV2C is enriched in the unusual dendrodendritic synapses of this area. In the glomeruli,

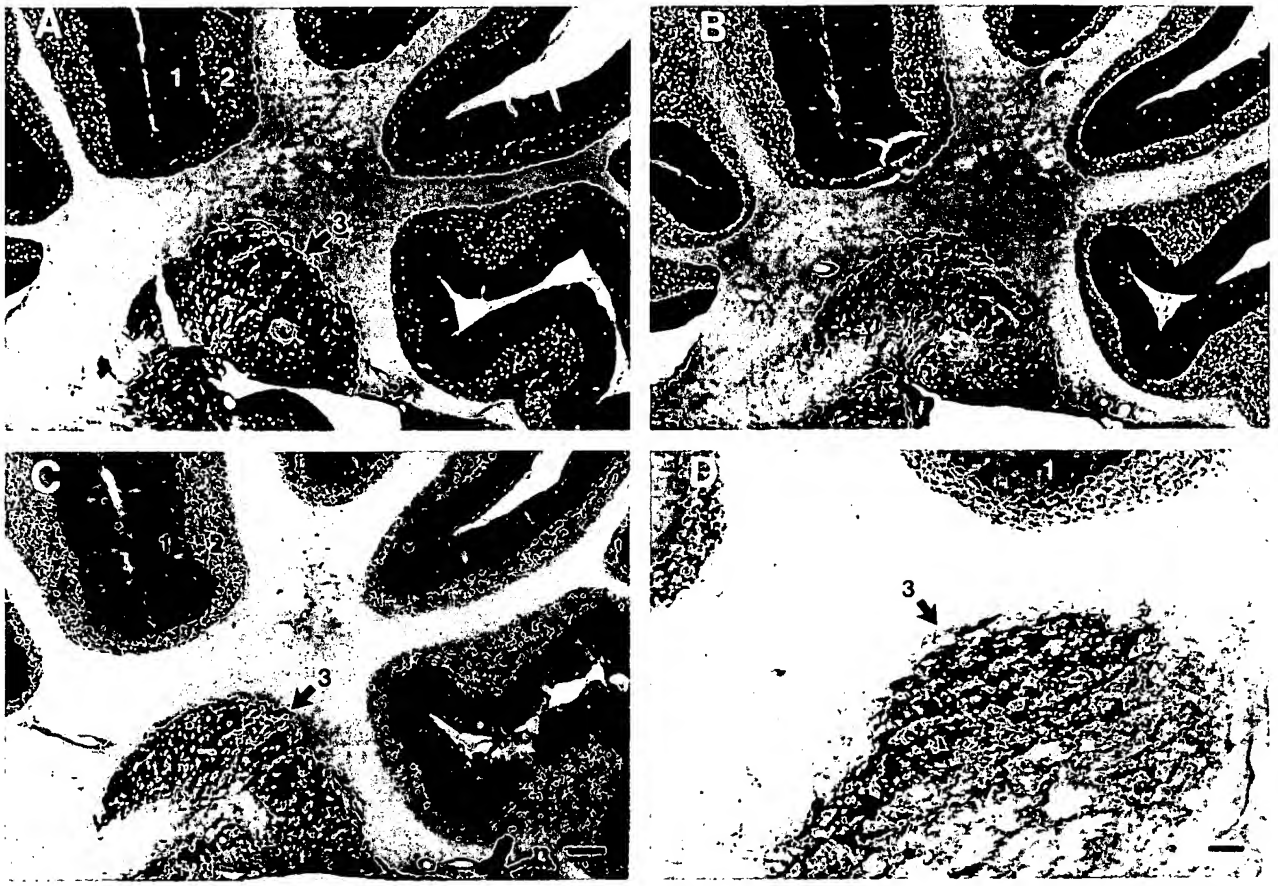

Fig. 11. Distribution of SV2 proteins in rat cerebellum. Sagittal sections of rat cerebellum were reacted with specific antibodies to SV2A (A), SV2B (B) and SV2C (C, D). D shows a higher magnification than C. Numbers identify the molecular layer (1) and the granule cell layer (2) of the cerebellum and the deep cerebellar nuclei (3). Scale bar = 0.1 mm (A–C), 50 μm (D).

SV2C was not equally distributed in all glomeruli like the other proteins. Instead, its levels varied between glomeruli (see long and short arrows in Fig. 13C). This remarkable staining pattern of SV2C in the olfactory bulb is specific, since the staining can be completely blocked with the peptide antigen used to generate the antibody (Fig. 13F).

DISCUSSION

SV2 is a synaptic vesicle protein that was discovered by a monoclonal antibody raised against purified vesicles.[7] Multiple studies revealed that SV2 is present on all synaptic vesicles, in addition to a number of secretory vesicles, suggesting that it may perform a fundamental function in membrane traffic.[2,7] The initial cloning of SV2 uncovered two distinct SV2 isoforms that react with the monoclonal SV2 antibody: SV2A and SV2B.[3] In the current study, we now show that a third SV2 protein called SV2C is expressed in mammalian brain. Like SV2A and SV2B, SV2C is primarily expressed in the brain, where it is highly enriched in synaptic vesicles. Furthermore, SV2C also reacts with the monoclonal SV2 antibody, indicating that all studies with this antibody actually dealt with a mixture of three distinct proteins. These findings suggest that the SV2 family consists of at least three highly homologous synaptic vesicle proteins in addition to the more distantly related vesicle protein SVOP.[16] Interestingly, although SV2 proteins are abundant components of vertebrate synaptic vesicles, no invertebrate ortholog for SV2 was uncovered in the recently completed *C. elegans* genome sequence or in *Drosophila melanogaster* sequencing projects. This indicates that SV2 is evolutionarily a relatively recent acquisition. This implies that there must have been a rapid gene duplication in order to achieve three SV2 isoforms in mammals with none in invertebrates.

Sequence analyses performed here revealed several remarkable properties of SV2 proteins. Among different members of the SV2 family, SV2A and SV2C are more similar to each other than to SV2B (Fig. 1). Furthermore, SV2C is also more homologous to the SV2 gene isolated from the elasmobranch *Discopyge ommata*[6] than SV2A and SV2B, suggesting that SV2C could be evolutionary more ancient than the other SV2 proteins. The high degree of conservation between the three SV2 proteins also suggests a common function. All SV2 proteins and the more distantly related SVOP are homologous to a number of transport proteins. Therefore, they may function as vesicular transporters in synaptic vesicles which use a proton gradient as driving force.[16] However, there is currently no firm idea what the transport substrate could be. In SV2 proteins, the presence of two conserved negatively charged amino acids in the first TMR indicates that the protein could transport a positively charged substrate. Therefore, SV2 is probably not transporting negatively charged substrates like chloride or ATP. The lack of any SV2 proteins in invertebrates suggests that such a transport

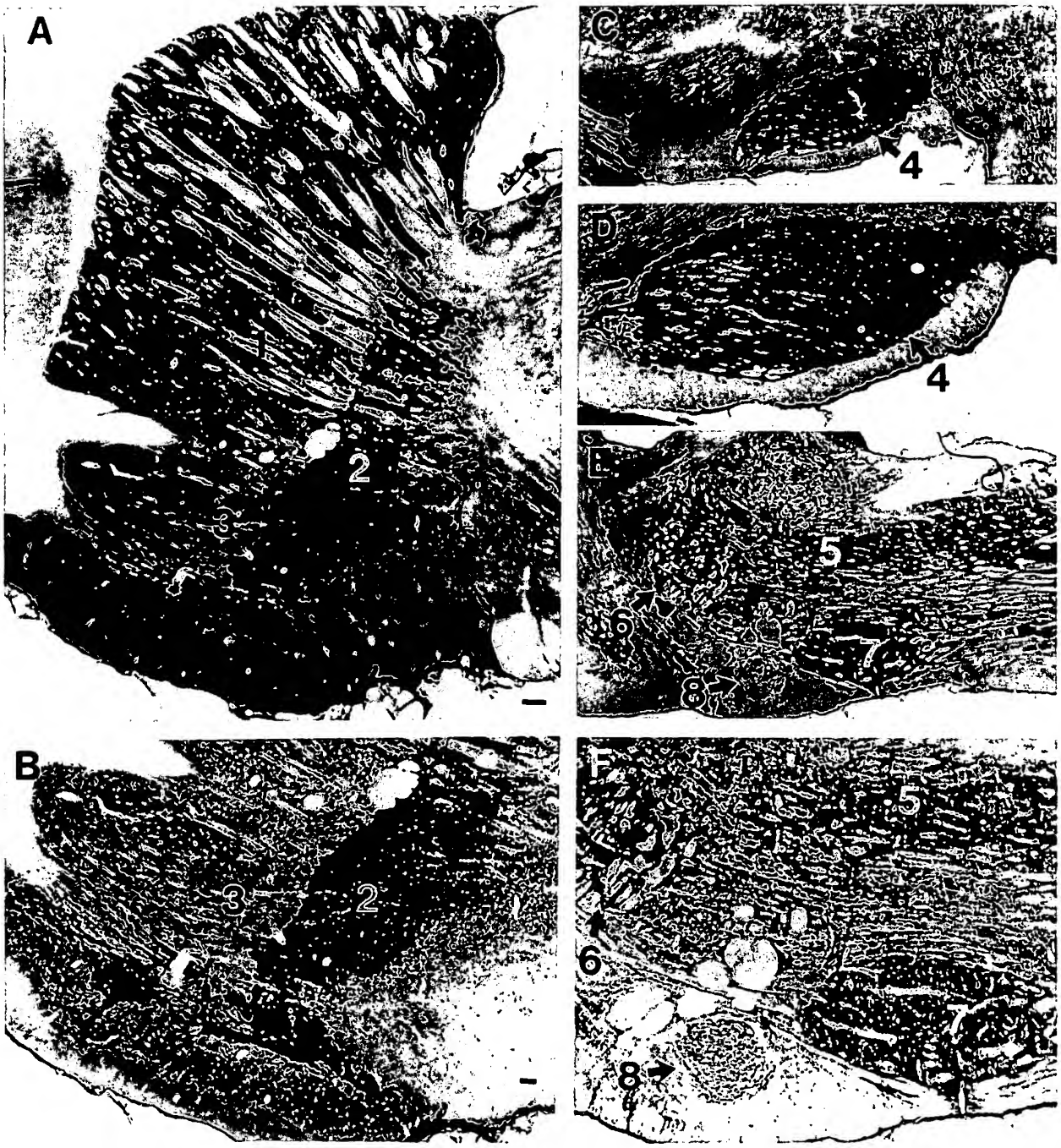

Fig. 12. Localization of SV2C in rat striatum (A, B), substantia nigra (C, D) and pons/medulla oblongata (E, F). Specific nuclei and areas are identified by numbers: putamen (1), pallidum (2), nucleus accumbens (3), substantia nigra (4), spinal trigeminus nucleus (5), motor trigeminal nucleus (6), nucleus fascialis (7), lateral superior olive (8). Scale bar = 0.2 mm (for all panels; bar in A applies to A, C and E, and that in B applies to B, D and F).

activity would not be required for basic functions of synaptic vesicles. An alternative hypothesis would be that SV2 proteins derived from transporter molecules, but now perform a different function on the vesicle. The conserved glycosylation of all isoforms and the remarkable structure of the domain between TMRs 7 and 8 makes a structural function of SV2 possible. It was recently described that the N-terminus of SV2A can interact with the C2B domain of the synaptic vesicle protein synaptotagmin. This makes an involvement of SV2A in the regulation of this calcium binding protein possible.[23] However, SV2B does not interact with synaptotagmin, probably due to the low degree of conservation in the N-terminus. It would be interesting to study if this interaction is also found with SV2C, which has a higher degree of conservation to SV2A.

Many synaptic vesicle proteins are expressed in differentially distributed isoforms; thus, the finding of very different localizations for SV2A, SV2B and SV2C is not surprising. What is surprising, however, is that when the various localizations of various vesicle protein isoforms are

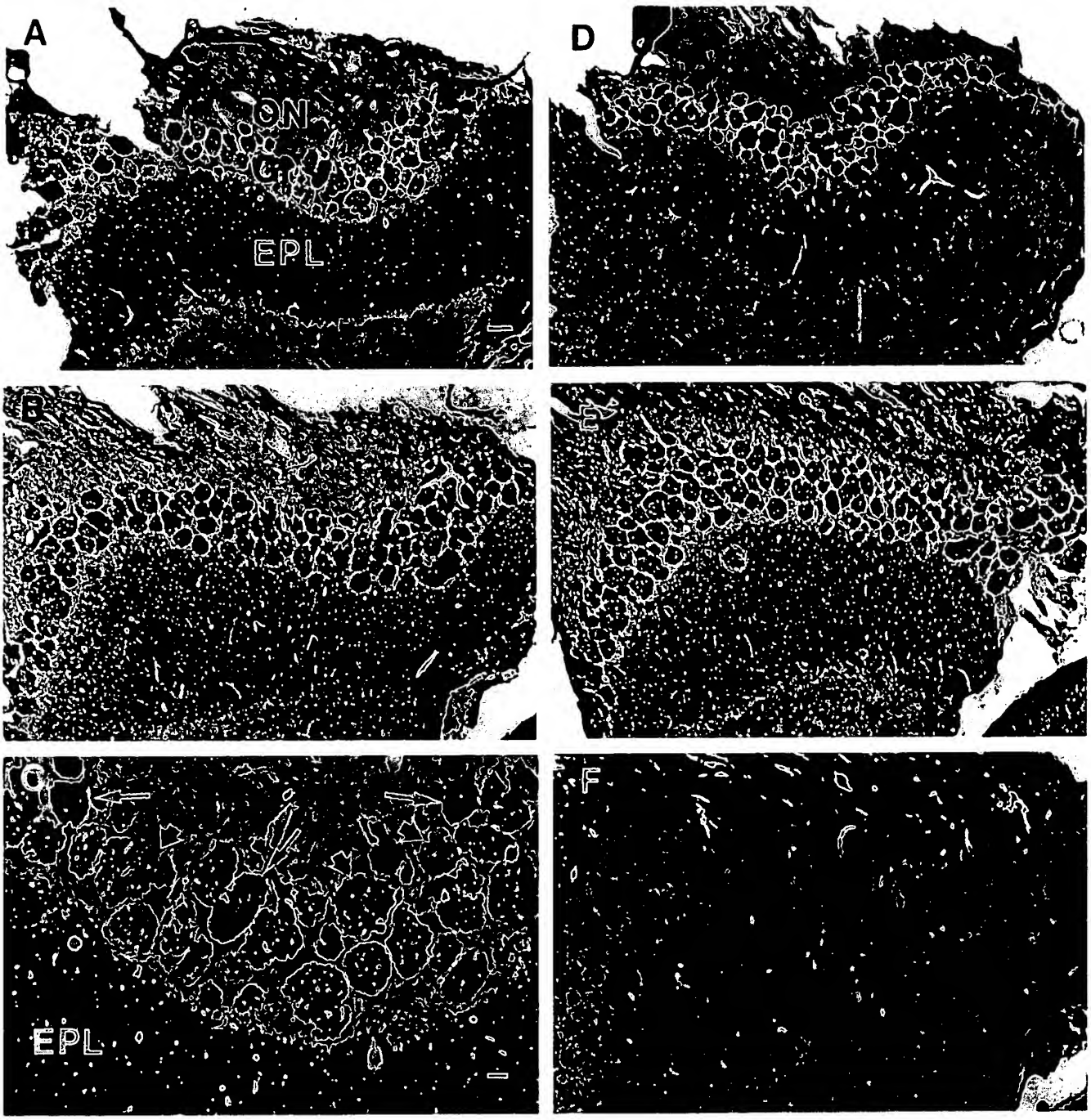

Fig. 13. Distribution of SV2 proteins in the olfactory bulb. Sagittal sections of rat olfactory bulb were reacted with specific antibodies to SV2C (A, C, F), SV2A (B), SV2B (E) and synapsins (D). In F, the SV2C antibody was blocked with the peptide used for the generation of the antibody as a specificity control. In A, the olfactory nerve layer (ON), glomerular layer (GL) and external plexiform layer (EPL) are identified. The long and short arrows in C label glomeruli, which show either strong or weak labeling, respectively, for SV2C. Scale bars = 0.1 mm (in A; applies to B, D–F), 50 μm (C).

compared with each other, they do not match. There is no common theme, no identifiable logic in the heterogeneous distributions of these proteins. For example, synaptotagmins I and II are expressed in a rostral/caudal gradient with synaptotagmin I concentrated in upper brain areas and synaptotagmin II in lower brain areas.[26] Synaptophysins, by contrast, are expressed in one ubiquitous isoform (synaptophysin I) and one isoform that is highly enriched in subsets of synapses, such as mossy fiber terminals (synaptophysin II/synaptoporin).[10] As a third example, four different synapsins are co-expressed in most neurons.[24] The pattern now observed for SV2 proteins is again different, with two isoforms being widely expressed and one isoform, the SV2C protein described here, very restricted. The only feature all of these differential distributions share is that none of them can be correlated with a particular functional feature (e.g., motor pathways or interneurons) or a particular neurotransmitter type.

The restricted distribution of SV2C is unlike any other

synaptic vesicle protein. It seems to be specifically excluded from cortical areas. SV2 proteins appear to be evolutionarily recent; of these, SV2C may be relatively older because its expression is concentrated in evolutionary older brain structures: the brainstem, basal ganglia and olfactory bulb. The question arises why three distinct SV2 isoforms with a high degree of sequence similarity evolved in mammals. It is possible that this occurred in order to allow for differences in the regulation of their expression, for example during development. An alternative explanation for the multiplicity of SV2 proteins is that each performs a different specific function in certain types of synapses.

CONCLUSIONS

In summary, in the current study we describe a novel isoform of SV2, SV2C, which is structurally very similar to the other SV2 isoforms but exhibits a strikingly different localization. The restricted presence of SV2C suggests that it could be useful as a specific synaptic marker in the study of degenerative diseases, like Parkinson's disease. The conserved architecture of the SV2 protein family in its multiple isoforms provides further clues to its function and suggests that any genetic studies on SV2 proteins will have to consider all isoforms.

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